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14. ABSTRACT

Resistance to chemotherapy presents challenges to prostate cancer management. Preliminary data suggest that Nanog is associated with prostate cancer stem cells and Nanog may cause resistance toward chemotherapy. The objective of this proposal is to define the role of Nanog in resistance of prostate cancer cells toward chemotherapy, to determine whether Nanog can be targeted to sensitize tumor cells toward chemotherapy, and to identify the downstream effectors,especially a family of efflux transporters, called, ATP-binding cassette transporters, in Nanog-mediated chemoresistance. Various molecular, cellular, and pharmacological approaches will be employed to achieve the goals of the proposed studies. The proposed studies will validate whether we can target tumor Nanog to sensitize prostate cancer stem cells toward chemotherapy so that tumor recurrence or drug resistance can be eliminated. By performing and achieving the goals of the proposed research, the principal investigator will learn how to conduct cutting edge research that can be of high significance in the translational cancer research.

15. SUBJECT TERMS
Nanog, cancer stem cells, chemoresistance, prostate cancer, efflux transporters

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Introduction

It is now increasingly accepted that cancer stem cells (CSCs, or tumor initiating cells) are responsible for tumor initiation. If cancer treatment kills most of cancer cells in the stage of transit amplifying and differentiation without killing the stem cells, the surviving cancer stem cells will eventually lead to recurrence of tumors. To eradicate cancer, we must learn more about the biology of cancer stem cells, their responses to treatments, and their role in tumor recurrence after treatment. In the preliminary studies, I found that Nanog, a transcription factor essential for self-renewal of embryonic stem cells, was expressed in prostate cancer cells, and further its expression was associated with tumor cells positive for stem/progenitor markers. Knockdown of Nanog reduced the ability of cancer cell to form tumors in an animal model. I further found that tumor cells with endogenous Nanog expression were particular resistant to chemotherapy. The data suggest that Nanog is associated with prostate cancer stem cells and Nanog may cause resistance toward chemotherapy.

Based on the preliminary data, it was hypothesized that Nanog promotes resistance of prostate carcinoma cells toward chemotherapy and that Nanog, or its downstream effectors, should be targeted for eradication of tumorigenic prostate carcinoma cells. To test my hypothesis, the following specific aims are proposed:

1) To define the role of Nanog in resistance of prostate carcinoma cells toward chemotherapy.

2) To determine whether Nanog can be targeted to eliminate the chemoresistance of prostate cancer cells.

3) To elucidate the mechanism of Nanog-mediated chemoresistance.

BODY OF REPORT

Scientific portion:

Task 1. To define the role of Nanog in resistance of prostate carcinoma cells toward chemotherapy. (Months 1 – 12).

Increased expression of Nanog in the surviving fractions of prostate cancer cells after chemotherapy: As a transcription factor essential for self-renewal of embryonic stem cells, Nanog has been found to be expressed in prostate cancer cells and further it regulates tumor development (1) and essential for prostate cancer cells to initiate tumor formation (Appendix 1). To determine whether Nanog plays a role in prostate cancer drug resistance, we first examined Nanog protein level in the surviving fractions of tumor cells after treatment with different chemotherapeutics. There was a higher level in Nanog protein in the surviving fractions of LNCaP cells treated with 10 nmol/L of Taxol, or 0.3 nmol/L vinblastine for 40 hours, when compared to those not treated (NA) or treated with DMSO (**Figure 1**, top panel). N-Tera cells were used as a positive control for western blot. In the surviving fractions of DU145 cells treated with vinblastine or doxorubicin, there were increased levels of Nanog protein (**Figure 1**, bottom panel). The results suggest that there were increased levels of Nanog protein in the surviving fractions of prostate cancer cells after chemotherapy.

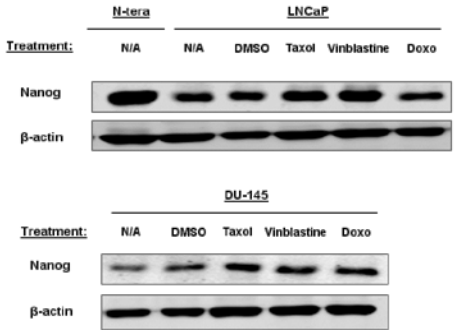

Figure 1. Increased Nanog protein level and promoter activities in the surviving fractions of prostate cells after chemotherapy shown by Western blot analysis. Note the increased Nanog levels in surviving fractions from Taxol or vinblastine treatment.

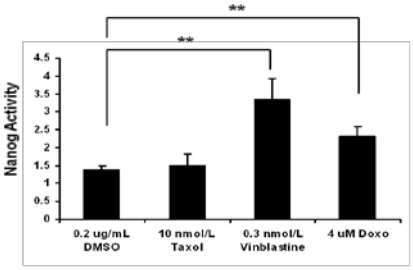

Figure 2. *NANOG1* promoter activities in DU145 cells after chemotherapy. The cells were transfected with *NANOG1* promoter luciferase reporter, and then treated with different therapeutic drugs. The surviving cells were harvested and assayed for luciferase activities. Note the increased NANOG1 promoter activities in cells treated with vinblastine or doxorubicin (N=3; ** $P < 0.01$).

Nanog has more than eleven pseudogenes (2). It has been suggested that the pseudogene *NANOGP8* is expressed at mRNA level in cancer cells (1), but one of our recent studies suggest that it is the *NANOG1* gene loci that is responsible for Nanog expression in tumorigenic prostate

cancer cells (3). To determine whether *NANOG1* promoter activities were increased after chemotherapy, LNCaP or DU145 cells were transfected with a *NANOG1* promoter luciferase reporter construct and then the cells were treated with different chemotherapeutics for 24 h and then the luciferase activities in the surviving fractions were assayed. As shown in **Figure 2**, the *NANOG1* promoter activities were increased in the DU145 cells after treatment with vinblastine or doxorubicin. The results suggest that the *NANOG1* promoter activities were either enriched in the surviving fractions of tumor cells after chemotherapy, or activated by treatment of chemotherapeutics.

Prospective enrichment of tumor cells with *NANOG1* promoter activities: To determine a possible functional role of Nanog in chemoresistance, we marked and selected tumor cells with active *NANOG1* promoter activities using a reporter construct in which expression of GFP and zeocin resistance is under the control of 2.5 kb *NANOG1* promoter (pGZ-Nanog). We enriched the cells with active *NANOG1* promoter activities using zeocin selection. After selection, most of zeocin-resistant cells transduced with pGZ-NANOG were GFP positive (**Figure 3**). When compared to the vector control, tumor cells enriched with active *NANOG1* promoter activities tended to form sphere-like structures (**Figure 3**). Western blot analysis revealed that the selection of cells with *NANOG1* promoter activities led to an enrichment of cells with higher endogenous NANOG expression at protein level (**Figure 4**), further suggesting a role of *NANOG1* in the endogenous expression of NANOG protein.

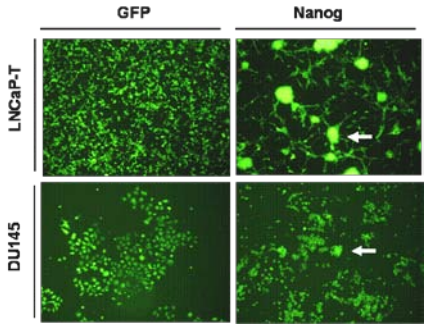

Figure 3. DU145 or LNCaP-T cells stably enriched with *NANOG1* promoter activities. Note the sphere-like structures in cells enriched with *NANOG1* (right panel), as compared to the vector control.

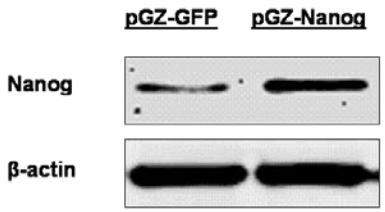

Figure 4. Western blot analysis of Nanog levels in Nanog-enriched cells vs. vector controls. Densitometry analysis revealed an approximate one fold increase in Nanog.

Tumor cells selected for active *NANOG1* promoter activities had increased expression of stem/progenitor markers: Normal prostate stem cells or prostate tumor stem cells have been identified as cells with high surface expression of integrin $\alpha_2\beta_1$, CD44, and CD133 (4) (5, 6). To determine whether Nanog expression marks a subpopulation of tumor cells with stem cell property, we analyzed the expression of stem cell markers $CD133^+/CD44^+$, in cells enriched for NANOG endogenous expression. An increase in CD133 protein levels in DU145 and LNCaP-T cells enriched with NANOG expression was found by Western blot (**Figure 5**). In addition,

increased CD44 surface expression was found in LNCAP-T cells enriched with NANOG expression as well as in DU145 cells enriched with NANOG expression (**Figure 6**).

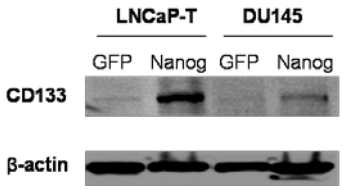

Figure 5. Increased levels of CD133, a marker of stem cells, in tumor cells with active NANOG1 promoter activities.

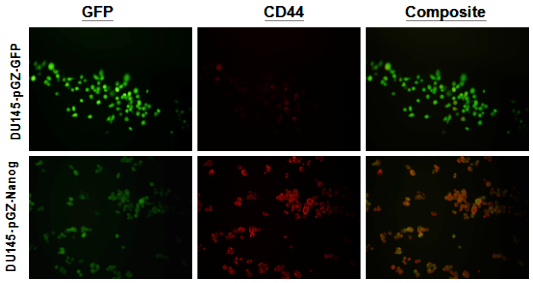

Figure 6. Increased levels of CD133 (Red color), a marker of stem cells, in tumor cells with active NANOG1 promoter activities.

Increased resistance toward chemotherapy by tumor cells with active *NANOG1* promoter activities: The above studies suggest that NANOG marks prostate cancer cells with stem cell markers. To determine whether tumor cells with endogenous Nanog expression are inherently resistant to chemotherapy, we evaluated the responses of Nanog-enriched cells toward several chemotherapeutics, in comparison with the vector control cells. As shown in **Figure 7**, the enriched Nanog-expressing LNCaP cells also presented an increased resistance toward taxol.

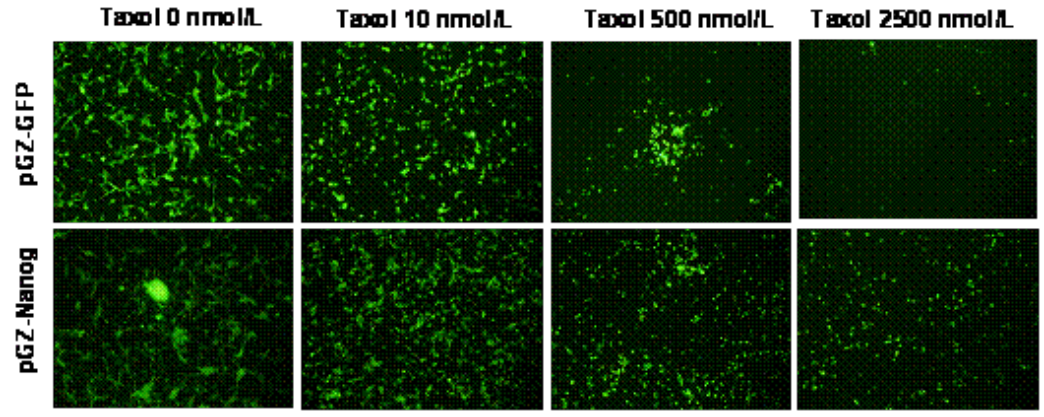

Figure 7. The cell morphology 24 h after taxol treatment: Most LNCaP-pGZ-GFP cells were sensitive to the taxol treatment at the concentration of 500 and 2500 nmol/L. The green cells indicate the remaining viable cells in culture after treatment.

As shown in **figure 8**, DU145 cells enriched with Nanog expression presented increased resistance to doxorubicin. The results suggest that enrichment of tumor cells with Nanog expression also enrich drug resistant cells. Here we propose to extend our preliminary studies on NANOG-mediated resistance toward chemotherapy.

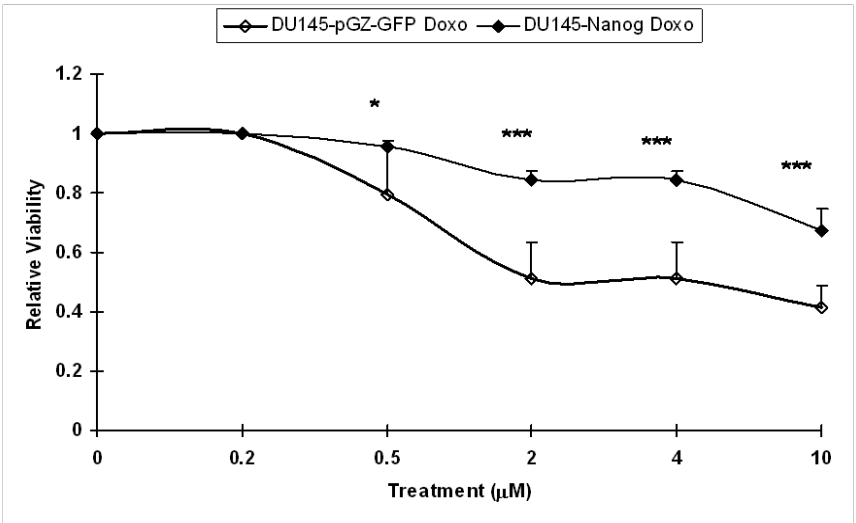

Figure 8. The Nanog-enriched cells exhibited enhanced resistance to doxorubicin in comparison with pGZ-GFP control cells. DU145-pGZ-GFP and –Nanog cells were treated with DMSO or doxorubicin for 72 hours, and the cell viability was measured by MTS assay. (N = 6; *** $P < 0.001$; * $P < 0.05$).

Forced expression of Nanog in prostate cancer cells: To determine whether Nanog expression is sufficient to render prostate cancer cells resistant to chemotherapy, I overexpressed Nanog in DU145 cells using a lentiviral vector. DU145 cells were infected with piPSC-hNanog or its vector control and the expression of Nanog was determined by Western blot analysis. As shown in **Figure 9**, DU145 cells infected with piPSC-hNanog had significantly increased Nanog protein. We are currently generating and characterizing stable sublines with Nanog stably overexpressed. Once we obtain them, we will evaluate whether forced expression of Nanog cause resistance toward chemotherapeutics.

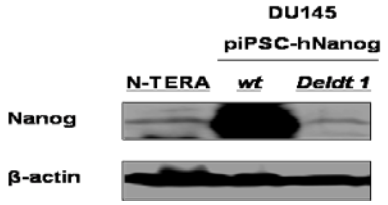

Figure 9. Validation of antibody used for Western blot analysis of Nanog protein. N-Tera cells were used as positive control. The antibody is validated by the observed large increase in Nanog protein levels after infection with the Nanog expressing virus (wt, middle panel), but not in cells infected with the vector control (Deldt1).

Task 2. To determine whether Nanog can be targeted to eliminate the chemoresistance of prostate cancer cells (Months 9 - 24).

To determine whether Nanog can be a target to reduce resistance toward chemotherapy, we attempted to knock down the expression of Nanog using small hairpin RNAs and examined the resultant effects on tumor cell responses toward chemotherapy. It was found that infection of DU145 cells with a shRNA construct targeting Nanog (7) led to a decrease in Nanog protein level (**Figure 10**). The knockdown of Nanog increased the sensitivity of DU145 cells toward Taxol (**Figure 11**), vinblastine (**Figure 12**) and doxorubicin (not shown).

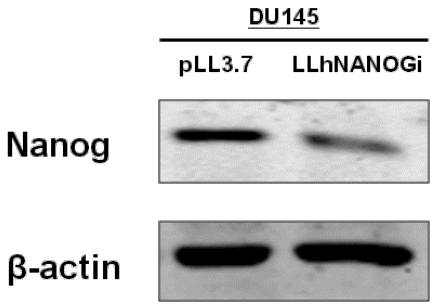

Figure 10. Western blot confirmation of the knockdown of Nanog in DU145 by shRNA.

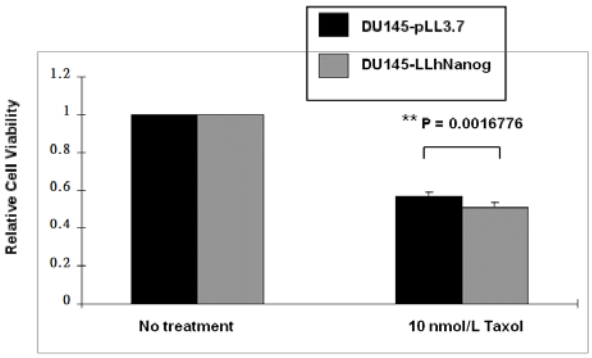

Figure 11. Increased sensitivity toward taxol by Nanog knockdown. The viability of surviving fractions was measured by MTS assay.

We further determined whether tumor cells with Nanog knocked down can be selectively eliminated by chemotherapy due to their increased chemosensitivity. Since the shRNA construct or its vector pLL3.7 utilized in Nanog knockdown also encodes GFP, we monitored the presence of cells with GFP positivity in the surviving fractions after chemotherapy. Parental DU145 cells were mixed with DU145 cells with Nanog knocked down or vector control cells, treated with different chemotherapeutics, and GFP positive cells in the surviving fractions were quantified by flow cytometry. If Nanog knockdown had no effects on the tumor cell sensitivity toward chemotherapeutics, we expect that GFP positive cells were still 50% in the surviving fractions. As shown in **Figure 13**, for DU145 cells infected with pLL3.7 (vector controls), there was a slight increase (more than expected 50%) in GFP positive cells in the surviving fractions. In contrast, in DU145 cells infected with Nanog knocking down LL-hNANOGi, there was a significant decrease in GFP positive cells in the surviving fractions after treatment with Taxol, vinblastine, or doxorubicin. The data suggest that tumor cells with Nanog knocked down were selectively eliminated. The increased sensitivity toward chemotherapy in tumor cells with Nanog knock down suggest an essential role for Nanog for tumor cells to resist chemotherapy.

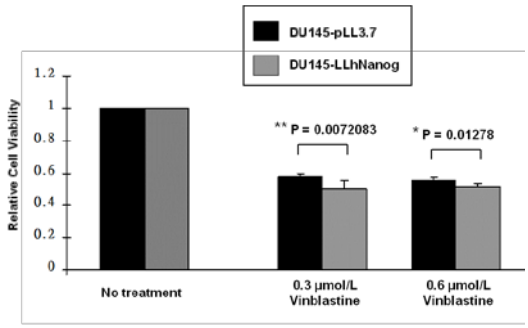

Figure 12. Nanog knockdown increased DU145 sensitivity toward vinblastine. Cell viability was measured by MTS assay.

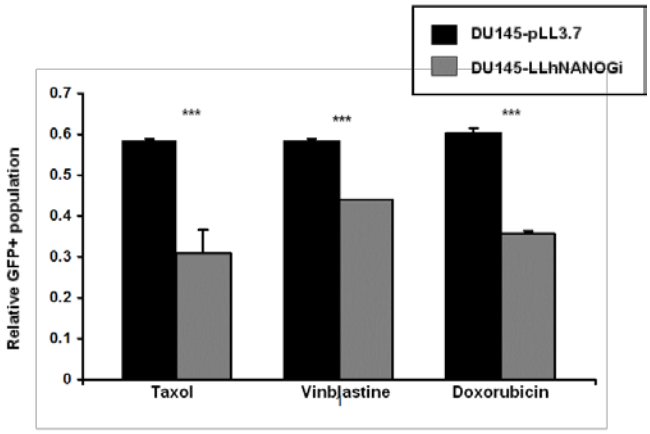

Figure 13. Selective elimination of Nanog knockdown cells by chemotherapy. Parental DU145 cells were mixed with GFP positive pLL3.7 DU145 cells or GFP positive LLhNANOGi DU145 cells in 1:1 ratio, and the mixed cells were treated with 0.2 µg/mL DMSO (as control), 10 nmol/L Taxol, 0.3 nmol/L Vinblastine as well as 6 µM Doxorubicin for 36 hours. The surviving cells were harvested for flow cytometry analysis for GFP positive cells (N = 3; *** P < 0.001).

Aim 3. To elucidate the mechanism of Nanog-mediated chemoresistance. (Months 18 - 36).

To determine the mechanisms involved in Nanog-mediated chemoresistance, we profiled genes involved in drug resistance, including drug metabolizing enzymes and transporters. As shown in **Figure 14**, MDR1 (ABCB1) and ABCG2 mRNA levels were significantly increased in DU145 cells enriched with Nanog1 promoter activities than in parental DU145 cells, while the expression of other ABC transporters were not significantly altered in DU145 cells enriched with Nanog expression.

Next we assessed the expression of MDR1 and ABCG2 at protein level. As shown in **Figure 15**, Western blot analysis revealed an increased level of p-glycoprotein (MDR1 or ABCB1) and ABCG2 in DU145 cells enriched with NANOG1 promoter activities, when compared to the parental control cells.

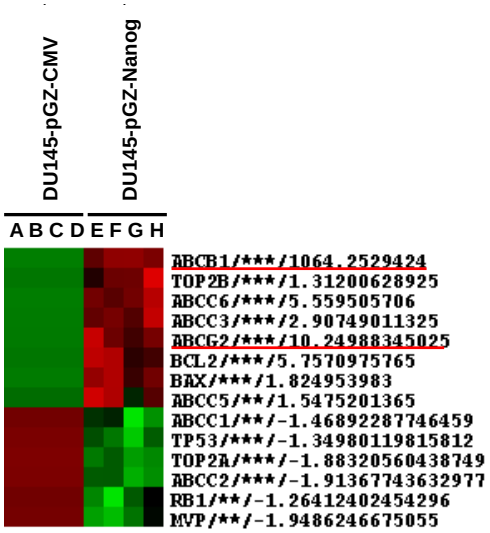

Figure 14. *Gene expression patterns in DU145 cells with increased Nanog levels.*

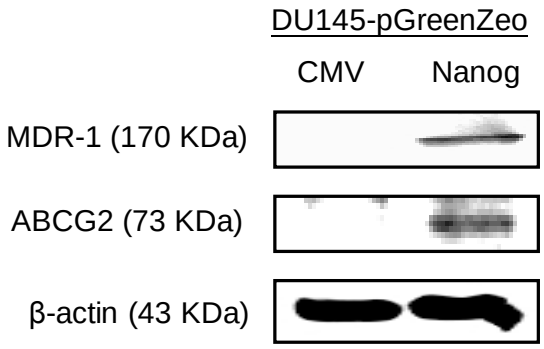

Figure 15. *Western blot analysis of MDR (ABCB1) and ABCG2 in DU145 cells enriched with Nanog expression.*

Immunocytochemical staining also revealed an increase in the surface expression of MDR1 as well as ABCG2 in DU145 cells enriched with NANOG1 promoter activities (**Figure 16**).

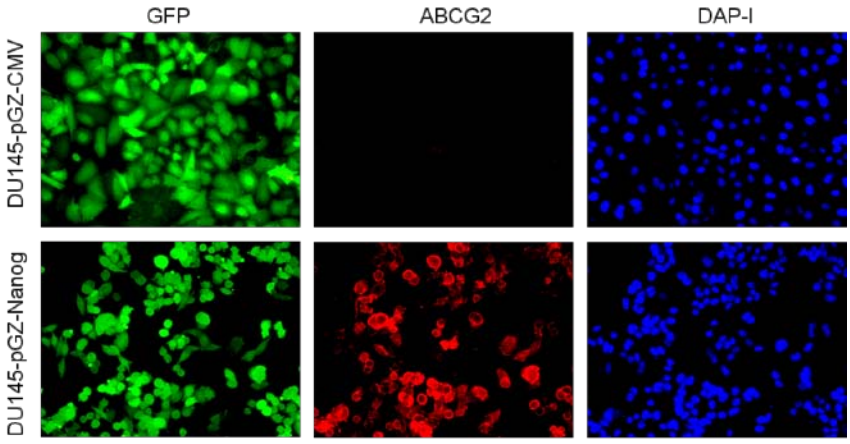

Figure 16. *Increased ABCG2 immunostaining in DU145 cells enriched with Nanog expression (DU146-pGZ-Nanog) when compared with control (DU145-pGZ-CMV).*

To further determine the role of Nanog in the expression of MDR1 and ABCG2 in prostate cancer cells, we depleted Nanog via shRNA and then examined the subsequent changes in these two ABC transporters. Nanog depletion reduced the levels of MDR1 and ABCG2 expression in prostate cancer cells (Data not shown here_. The data further confirm the regulation of MDR1 and ABCG2 expression by Nanog.

Roles of MDR1 and ABCG2 in Nanog-mediated resistance

The increased expression of MDR1 and ABCG2 may confer the Nanog-expressing tumor cells with increased resistance toward chemotherapy. First we examined the effects of an inhibitor of ABCG2, FTC, on Nanog-mediated resistance. As shown in **Figure 17**, FTC treatment reduced, but not abolished, the resistance of Nanog-expressing cells toward vinblastine

and doxorubicin, suggesting a partial contribution of ABCG2 in resistance of Nanog-expressing cells toward chemotherapeutics.

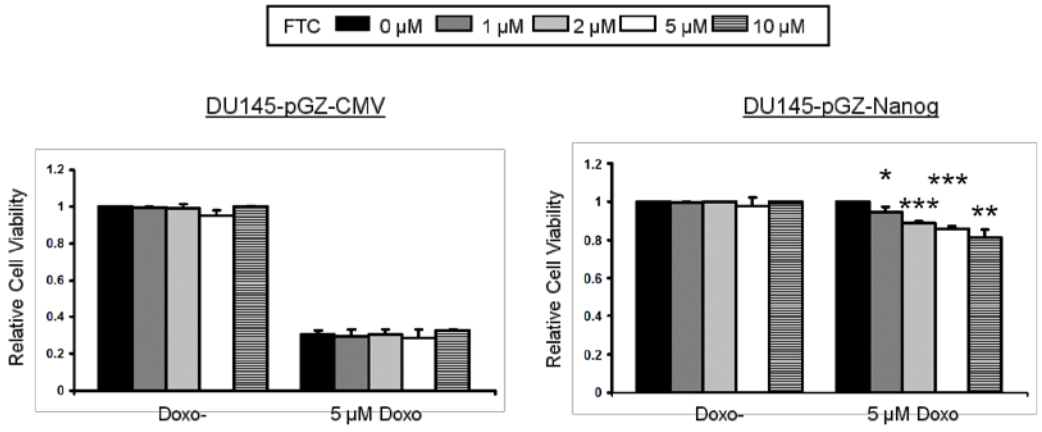

Figure 17. *ABCG2 inhibitor FTC attenuated, but not abolished, the chemoresistance mediated by Nanog toward doxorubicin.*

To determine whether MDR1 (ABCB1) also played a role in the Nanog-mediated resistance toward chemotherapy, we examined the effects of UIC2, a neutralizing antibody of MDR1, on Nanog-mediated resistance. As shown in **Figure 18**, UIC2 pretreatement reduced the increased resistance of DU145-Nanog cells toward Taxol. UIC2 pretreatment had minimal effects on the responses of DU145-CMV toward Taxol.

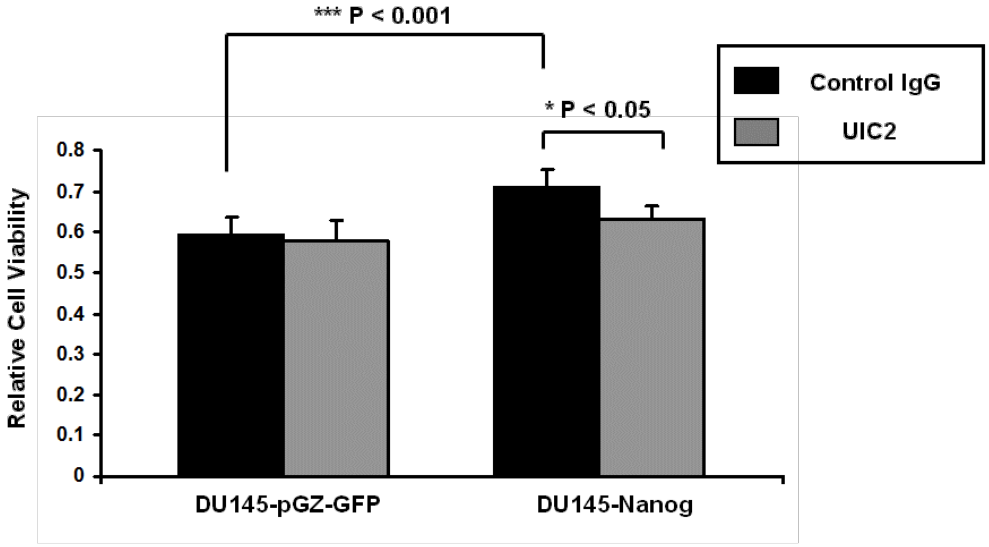

Figure 18. *MDR1 neutralizing antibody UIC2 reduced the increased resistance of DU145-pGZ-Nanog cells toward Taxol.*

To further test the role of MDR1 in Nanog-mediated resistance, we examined the effects of MDR1 depletion on Nanog-mediated resistance. As shown in **Figure 19**, depletion of MDR1 drastically reduced the resistance of Nanog-expressing cells toward Taxol and vinblastine, and to lesser extent doxorubicin, as evidenced by the reduced colony formation. The data suggest an important role of MDR1 in Nanog-mediated resistance.

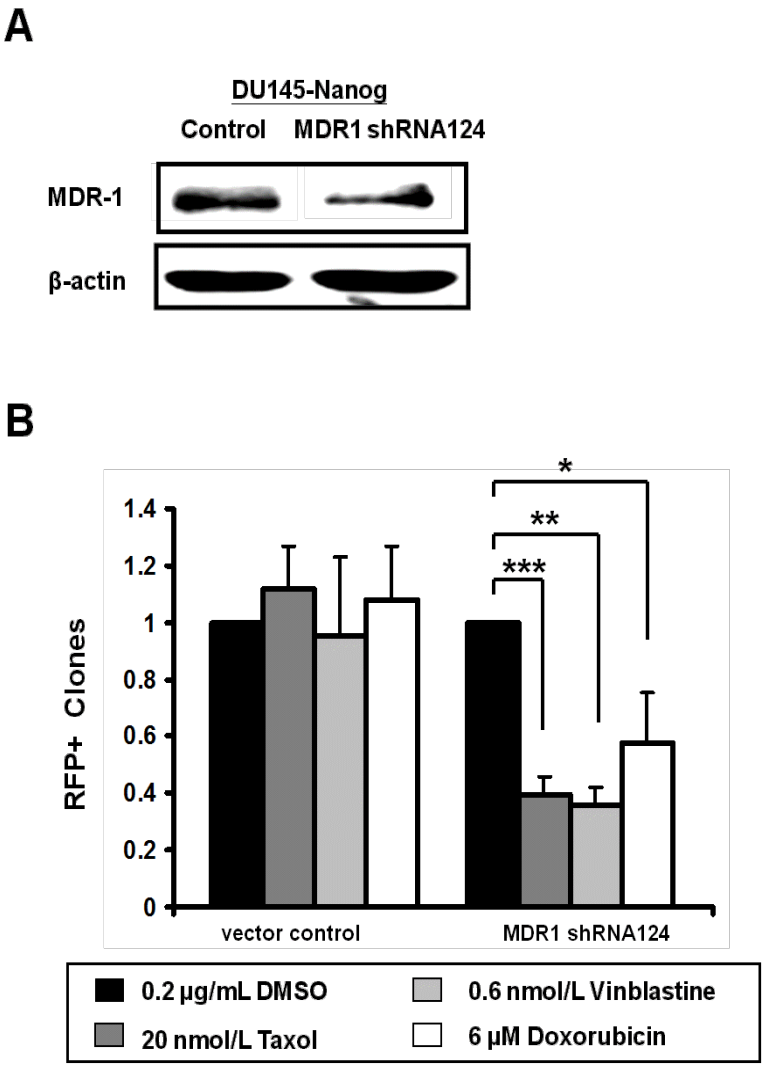

Figure 19. Depletion of MDR1 by shRNA sensitized DU145 cells enriched with Nanog toward chemotherapy. A, Western blot analysis of pepletion of MDR1 by shRNA. B. Reduced colony formation of DU145-pGF-Nanog cells after depletion of MDR1 in responses to chemotherapy. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The above data collectively suggest that the increased chemoresistance by DU145 cells enriched with Nanog activities is mediated by ABCG2 and MDR1.

Training portions

In the last year, the original PI, Man-Tzu Wang, obtained her Ph.D. degree. Now she is a postdoctoral fellow in Dr. Frank McCormick lab at UCSF Cancer Center.

The new PI, Ms. Hongmei Jiang, has had the following trainings:

A. Research-related training by learning all laboratory techniques required to complete the proposed studies, including, but not limited to: (Month 5 – 36)

Extraction of large plasmids more than 10 kb, cell culture, packaging of viral vectors, generation of stable cell lines with Nanog expressed or knocked down, FACS, evaluation of tumor cell responses to chemotherapy using MTS, trypan blue exclusion, and colony formation assays, Western blot, RNA isolation and cDNA synthesis, cloning, site-directed mutagenesis, and statistical analysis.

B. Non-research tasks important for PI's career development:

Ms. Jiang attended career development workshops sponsored by the 2013 AACR Annual Meeting held in Washington, DC in April, 2013.

B1. Oral presentations:

1) Presentations of research progresses in the lab meeting weekly. Ms Jiang has presented research findings in the lab meeting on weekly basis.
2) Presentations at student seminars. Ms. Jiang has given a seminar on her research findings in the spring. The audience is made up with students in the MBMB programs, faculty, and other interested researchers.
3) Presentation at scientific meetings. Ms Jiang and Wang presented the research findings in 2011 IMPACT meeting sponsored by DoD PCRP. Ms also Jiang presented the research findings in 2013 AACR Annual Meeting.

B2. Scientific writing skills:

1) Writing and submission of the annual progress report to DoD. (Every year)
2) Writing of research protocols or experimental approaches. (Every year)
3) Writing the first draft of manuscript to be submitted (2013- present)
4) Writing the first several chapters of dissertation (Dec. 2013 – present)

KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

Presentations:

Man-Tzu Wang and Daotai Nie. Nanog, cancer stem cells, and resistance to chemotherapy. 2011 DoD PCRP Impact Meeting, Orlando, March 2011.

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. NANOG promotes chemoresistance in prostate carcinoma cells. AACR Annual Meeting, Chicago, March 31 – April 4, 2012.

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. The Role of POU5F1B in Prostate Cancer. AACR 2013 Annual Meeting, Washington, DC, April 2013.

Hongmei Jiang, Man-Tzu Wang, and Daotai Nie. The Role of POU5F1B in Prostate Cancer. Simmons Cancer Institute 2013 Research Symposium, Springfield, IL, October 2013.

Abstracts published:

Man-Tzu Wang and Daotai Nie. Nanog, cancer stem cells, and resistance to chemotherapy. Proceedings of the 2011 DoD PCRP Impact Meeting.

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. Nanog, NANOG promotes chemoresistance in prostate carcinoma cells. Proc. Amer. Assoc. Cancer Res. 51 Late breaking: LB-292, 2012.

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. The Role of POU5F1B in Prostate Cancer. Proc. Amer. Assoc. Cancer Res. 52 Late breaking: LB-281, 2013.

Review article published:

Man-Tzu Wang, Hongmei Jiang, Debasish Boral and Daotai Nie. Cancer Stem Cells in Resistance to Cytotoxic Drugs: Implications in Chemotherapy. B. Bonavida (ed.), Molecular Mechanisms of Tumor Cell Resistance to Chemotherapy, Resistance to Targeted Anti-Cancer Therapeutics 1, DOI: 10.1007/978-1-4614-7070-0_8, Springer Science+Business Media New York 2013.

Research articles published:

The manuscript is in the process of revisions.

Conclusions and significance (So what?):

Identification of key factors for tumor resistance to chemotherapy can lead to better strategy in cancer treatment. Our studies suggest that Nanog, a transcription factor essential for the self-renewal of embryonic stem cells, is expressed in tumorigenic cancer cells and further Nanog expression was enriched in the surviving fractions of tumor cells after chemotherapy. Knockdown of Nanog sensitized prostate cancer cells toward chemotherapy. Our studies suggest that Nanog can be targeted to improve the efficacy of chemotherapy of prostate cancer.

APPENDICES

N/A

SUPPORTING DATA

Embedded in the reporting body

REFERENCES

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